PCT/EP2004/003662
01.04.04

Europäisches Patentamt
European Patent Office
Office européen des brevets

Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03100896.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Europäisches Patentamt | European Patent Office | Office européen des brevets

Anmeldung Nr:
Application no.: 03100896.4
Demande no:

Anmeldetag:
Date of filing: 04.04.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

DSM IP Assets B.V.
Het Overloon 1
6411 TE Heerlen
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Fermentation processes using low concentrations of carbon- and nitrogen-containing nutrients

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12P1/04
C12R1/01
C12R1/04
C12R1/465
C12P19/62

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT SE SI SK TR LI RO

An improved composition for fermentation processes

Field of the invention

The present invention relates to the field of fermentative production of desired compounds, such as secondary metabolites, proteins or peptides.

Background of the invention

The actinomycetes, the family of filamentous bacteria, are of great importance for the fermentation industry. Many members of this family are known, which are producers of secondary metabolites or producers of extracellular enzymes, and several of these products of bacterial metabolism have found an industrial application. For obtaining these products, the bacteria are generally cultivated in liquid media (submerged cultures), leading to excretion of the products in the liquid, from which they can be isolated. Formation of product can take place during the initial fast growth of the organism and/or during a second period in which the culture is maintained in a slow-growing or non-growing state. The amount of product which is formed per unit of time during such a process (the productivity) is generally a function of a number of factors: the intrinsic metabolic activity of the organism, the physiological conditions prevailing in the culture (e.g. pH, temperature, medium composition), and the amount of organisms which is present in the equipment used for the process. Generally, during optimization of a fermentation process, it will be tried to get a concentration of bacteria that is as high as possible, because then the highest titer of product will be obtained, assuming that the intrinsic productivity per unit of organism is a constant. One particular characteristic of bacteria belonging to the family of actinomycetes makes it difficult to achieve this goal. Actinomycetes, when grown in submerged culture, have a filamentous morphology, which generally leads to highly viscous culture fluids. A high viscosity of the culture limits the rate of oxygen transfer to the culture. Virtually all processes with actinomycetes depend on the presence and consumption of oxygen, therefore a limitation in oxygen transfer will impose a limitation on the overall process productivity. Viscosity of a culture fluid is determined by a number of factors such as the composition of the medium, the

presence and nature of products excreted by the microorganisms, and (most important) the morphology of the microorgansm. If one would be able to influence the morphological characteristic in a positive way (i.e. to decrease the specific viscosity), this would give the following possibilities to improve a given fermentation process: the same process could be operated at a higher production rate or it would be possible to achieve a higher concentration of bacteria. Both changes in the process will result in higher process productivity.

Summary of the invention

The present invention discloses a fermentation process in liquid medium for the production of a desired compound wherein a filamentous bacterial strain is cultivated in a fermentation medium in which carbon containing nutrients and nitrogen containing nutrients are maintained at low concentrations in the fermentation medium.

Preferably in this fermentation process the feed is supplied comprising carbon and nitrogen containing nutrients, in such a ratio that low concentrations of both carbon and nitrogen containing nutrients are maintained in the culture.

The filamentous bacteria are preferably of the family Actinomyces, more preferably of the genus Streptomyces.

Detailed description of the invention

Surprisingly we have found that certain medium compositions lead to a reduced culture viscosity of a fermentation process using a filamentous bacterial strain, without affecting the production of the desired compound. An important factor appeared to be the ratio of nitrogen containing nutrients to carbon containing nutrients in the medium. A high N/C ratio (relative excess of nitrogen compounds) leads to viscous cultures, whereas a low N/C ratio resulted in relatively low viscosity of the culture fluid. When the amount of nitrogen in the medium is restricted too much, this leads to very poor growth of the organism and low amounts of product formed. However, at an intermediate N/C ratio, growth of the organism is still good and product formation is normal, while the morphology of the organism is apparently changed in such a way that the viscosity of the culture fluid is significantly reduced. The consequence of this finding is, that by carefully controlling the medium, or more specifically: by controlling the ratio of carbon

and nitrogen containing nutrients in the medium, a process with this type of organism can be improved significantly.

Strains of the family Acinomycetes, Acementous bacteria are known to produce desired compounds like secondary metabolits proteins and peptides which have commercial applications. Examples hereof are natamycin, nistatine, glucose isomerase, clavulanic acid.

For example, the actinomycetes *Streptomyces natalensis* and *Streptomyces silvosporens* produce the antifungal compound natamycin, which has several applications as antifungal compound. Fermentation processes of such filamentous bacteria are generally characterised by two phases. Usually the process starts with a phase where growth of the microorganism occurs until conditions for growth become nfavourable, for instance because one of the growth supporting nutrients becomes depleted from the medium. The initial (batch) phase may be followed by a phase where the microorganisms are maintained in a viable state. Often most of the product of interest is formed in this second phase. In this second phase, more nutrients may be supplied to the culture, either discontinuously as a single or repeated charge of fresh nutrients, or continuously by feeding one or more nutrients containing fluids to the fermentation vessel. This mode of fermentation is called fed-batch fermentation. Preferably a fermentation process may be further prolonged by taking out a part of the fermentation mash, for instance when the fermentation vessel becomes completely filled as a result of feeding of nutrient containing fluids. This process form is called extended fermentation or repeated (fed-)batch fermentation. The first phase will end in case one of the nutrients is depleted which can be measured by following the oxygen uptake which will decrease at the end of the first phase. In general the first phase will take 6 to 48 hours, The second phase starts when feeding of the nutrients is started. Feeding of nutrients allows the continuation of the process for a longer period than is possible in simple batch fermentation process. In general, for each production process, the most optimal ratio of carbon and nitrogen can be determined by the skilled person, depending on the elementary composition of the organism and the product(s), and depending on the effect of the N/C ratio on the physiology of the organism, more specifically, the product forming capacity of the organism. We have found that neither carbon excess nor nitrogen excess will lead to the desired result. In the optimum situation, both the available carbon and nitrogen will be almost depleted from the medium, at the end of the batch process, and/or during the process in a prolonged fed-batch type fermentation

process. The concentration of the medium in the second phase is therefore preferably below 0.5 g/l for the nitrogen containing nutrient. For the carbon containing nutrient preferably this concentration is below 1 g/l. The feed can be supplied via one feed containing all the nutrients , or preferably via more than one subfeeds each comprising either nitrogen containing nutrient, carbon containing nutrients or a combination of nitrogen and carbon containing nutrients.

In case the optional ratio between C and N is determined the feed is controlled in such a way that the amount of oxygen is between 20 and 70% of air saturation, preferably between 30 and 60% of air saturation.

Oxygen, in general in form or air, is in general introduced at or near the bottom of the fermentor. One of more nozzles are installed for the introduction of air or another containing gas including (purified) oxygen.

Optionally a stirrer is present in the reactor to stimulate the oxygen uptake. Moreover the stirrer prevents concentration gradients of the feed subfeeds in the fermentor.

Legend to the Figures

Figure 1: Viscosity development of a nitrogen excess-culture (•) and a nitrogen-carbon double-limited culturs (♦).

Figure 2: Agitation power required to control the dissolved oxygen concentration at a 30% air saturation. Both cultures, nitrogen excess (•) and nitrogen-carbon double-limited (♦) were operated under otherwise similar process conditions.

Figure 3: Viscosity development of a nitrogen excess culture (•) and a nitrogen-carbon double-limited culture (♦).

Figure 4: Product accumulation in a nitrogen excess culture (•) and a nitrogen-carbon double-limited culture (♦).

Figure 5: Full scale fermentations of *Streptomyces natalensis* producing natamycin. The initial process (•) used a limiting feed of soybean oil, while the NH3 concentration was kept at a non-limiting level. In the improved process (♦) the NH3 concentration was kept at a low value by continuous feeding of a NH3 solution in proportion to the oil feeding rate. The reduced culture viscosity allowed faster feeding of oil. The increase in product formation was approximately proportional to the increase in oil feeding rate.

EXAMPLES

Example 1

Steptomyces natalensis strain ATCC27448 was cultivated in conical shake flasks of 2000 mL maximum content, containing 500 mL growth medium of the following composition:

	g/L
Glucose.1H_2O	30
Casein hydrolysate	15
Yeast Extract (dried)	10
De-foamer Basildon	0.4

The pH was adjusted to 7.0 by NaOH/H_2SO_4, and the medium was sterilized by autoclavation (20 minutes at 120°C). The content of a full-grown shake flask was used to inoculate a fermentation vessel, containing 6-liter medium of the following medium:

	g/L
Soybean flower	25
Soybean oil	8
Corn Steep (dried)	1
KH_2PO_4	0.45
Trace elements solution	17
De-foamer Basildon	0.4

The composition of the trace elements solution was as follows:

	g/L
Citric acid.1H_2O	175
$FeSO_4$.7H_2O	5.5

$MgSO_4.7H_2O$	100
H_3BO_3	0.06
$CuSO_4.5H_2O$	0.13
$ZnSO_4.7H_2O$	1.3
$CoSO_4.7H_2O$	0.14

The temperature and pH of the medium were controlled at 25°C and 7.0 respectively. Dissolved oxygen concentration was kept above 30% of air saturation, by increasing airflow and/or stirrer speed when necessary. After preliminary growth in batch culture during approximately 24 hours the culture entered a second phase of the fermentation, where growth and product formation were continued by feeding pure soybean oil. A second feeding line was installed to feed ammonia. The average feeding rate of the soybean oil was 3 g/h. Ammonia was supplied in proportion to the soybean oil feeding rate. A series of fermentations was carried out, in which different ammonia feeding rates were applied, keeping the soybean oil feading rate constant. For this strain it was found that both the carbon source and the nitrogen source were totally consumed when the ratio of NH3 to oil was in the range of 30-40 mg NH3/g oil. This condition of C-N double limitation resulted in cultures with the lowest specific viscosities. Nitrogen excess (NH3/oil ratio >40 mg/g) resulted in a considerable increased viscosity of the culture. Carbon excess (NH3/oil ratio <30 mg/g) had a similar effect. In addition, the accumulation of oil had a negative effect on the culture viability. The range of ratios of nitrogen containing nutrients versus carbon containing nutrients is dependent on the strain and the nature of the nitrogen and carbon sources. For every new process, the optimal range can therefore be determined by the present procedure.

Two experiments were done according to the process description of example 1. One experiment was aimed to reach a condition of nitrogen excess (i.e. the culture is then purely limited by the soybean oil feeding rate). In another experiment the rate of ammonia feeding relative to soybean oil feeding was reduced, in order to arrive at a condition where the concentration of both nutrients (soybean oil and ammonia) in the fermenter vessel is very low. For the test organism in the chosen conditions it was found that the ratio of ammonia feeding rate relative to the oil-feeding rate should be around 35 mg NH_3 per g oil. The ammonia surplus experiment was carried out at a ratio of 45 mg NH_3 per g oil.

The effect of the carbon-nitrogen double limitation is clearly demonstrated in Figure 1. Under nitrogen excess conditions the viscosity reaches the usual high values. Under conditions of simultaneous carbon and nitrogen limitation, the viscosity drops to a much lower value, causing better aeration conditions. For a good production it is preferred that the dissolved oxygen concentration is maintained at a level of above 30% of air saturation. Figure 2 illustrates that for maintaining this dissolved oxygen concentration much less agitation power is needed when the culture is under a condition of nitrogen-carbon double limitation.

Example 2

Another fermentation experiment was done using the same procedure as described for example 1 using a strain of *Streptomyces natalensis* This strain is a producer of anti-fungal compound natamycin. In this experiment two fermentations were run. One experiment was under carbon limitation and nitrogen excess (NH_3 level was kept at 150-200 mg/L during the oil feeding phase). The second experiment was run under nitrogen-carbon double limitation during the oil feeding phase, employing a NH_3/oil ratio of 32 mg/g. Some results are shown in Figure 3 and 4. It is obvious that also for this strain a very significant difference in viscosity is found between the two modes of fermentation. A low viscosity is very benifical for efficient process operation. However when the conditions that result in a low viscosity would result in a poor product formation potency, the over-all effect would be negative. In this experiment it was found that product formation is not affected at all by the conditions leading to low viscosity (Figure 3). Even it seems that product formation in the nitrogen-carbon double limitation experiment is faster in the second part of the fermentation, after a slightly slower start.

Example 3

The information obtained in experiments like described in examples 1 and 2 was used to improve the actual production process of natamycine at full scale ($100m^3$ scale). The reduced viscosity allows intensification of the process by faster feeding of the main nutrient soybean oil. The feeding rate of NH3 was proportional to the feeding of oil, as described in the examples 1 and 2, resulting in carbon-nitrogen double limitating during the feeding phase (which started at about 24 hours after inoculation of the fermentation vessel). Process conditions and medium composition were similar to the small scale experiments described in example 1 and 2. Starting with a small increase, the oil feeding

rate was increased step-wise from run to run, until such a process intensity was reached that the criterion on minimal dissolved oxygen tension could just be maintained. As figure 5 illustrates, the improvement in product output as result of the higher oil feeding rate was quite substantial.

5

CLAIMS

1. A fermentation process in liquid medium for the production of a desired compound wherein a filamentous bacterial strain is cultivated in a fermentation medium in which carbon containing nutrients and nitrogen containing nutrients are maintained at low concentrations in the fermentation medium.
2. A process according to claim 1, wherein a feed is supplied comprising carbon containing nutrients and nitrogen containing nutrients, in such a ratio that low concentrations of both carbon and nitrogen containing nutrients are maintained in the culture.
3. A process of claim 1 or 2, wherein the feed is supplied to the fermentation process via more than one subfeed, each subfeed comprises either nitrogen containing nutrients, carbon containing nutrients, or a combination of nitrogen and carbon containing method.
4. A process according to any one of claim 1-3, where in from the moment that the feed is supplied, the concentration of the nitrogen containing nutrient in the medium is below 0.5 g/l (expressed as gram nitrogen per liter), and where in the concentration of the carbon containing nutrient in the medium is below 1 g/l (expressed as gram carbon per liter).
5. A process according to any one of claims 1-4 wherein the amount of oxygen is between 20 and 70% or air saturation, preferably between 30 and 60% of air saturation.
5. The process as in claim 1-4, using bacteria of the family of *Actinomycetes*.
6. Any process as in claim 1-5, using a bacterium of the genus *Streptomyces*.
7. Any process as in claim 1-6, using the bacterium *Streptomyces natalensis* or *Streptomyces gilvosporeus*, and where the desired compound is natamycin.

An improved composition for fermentation processes

ABSTRACT

The present invention describes a fermentation process in liquid medium for the production of a desired compound wherein a filamentous bacterial strain is cultivated in a fermentation medium in which carbon containing nutrients and nitrogen containing nutrients are maintained at low concentrations in the fermentation medium.

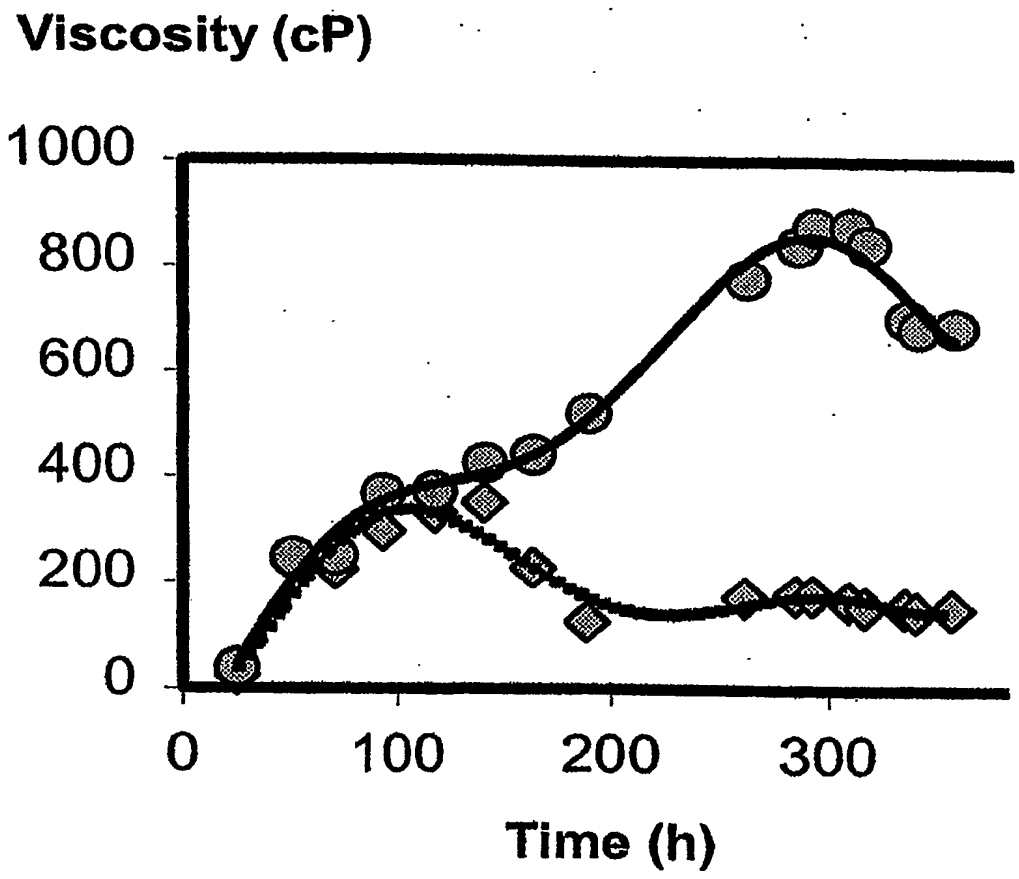

Fig. 1

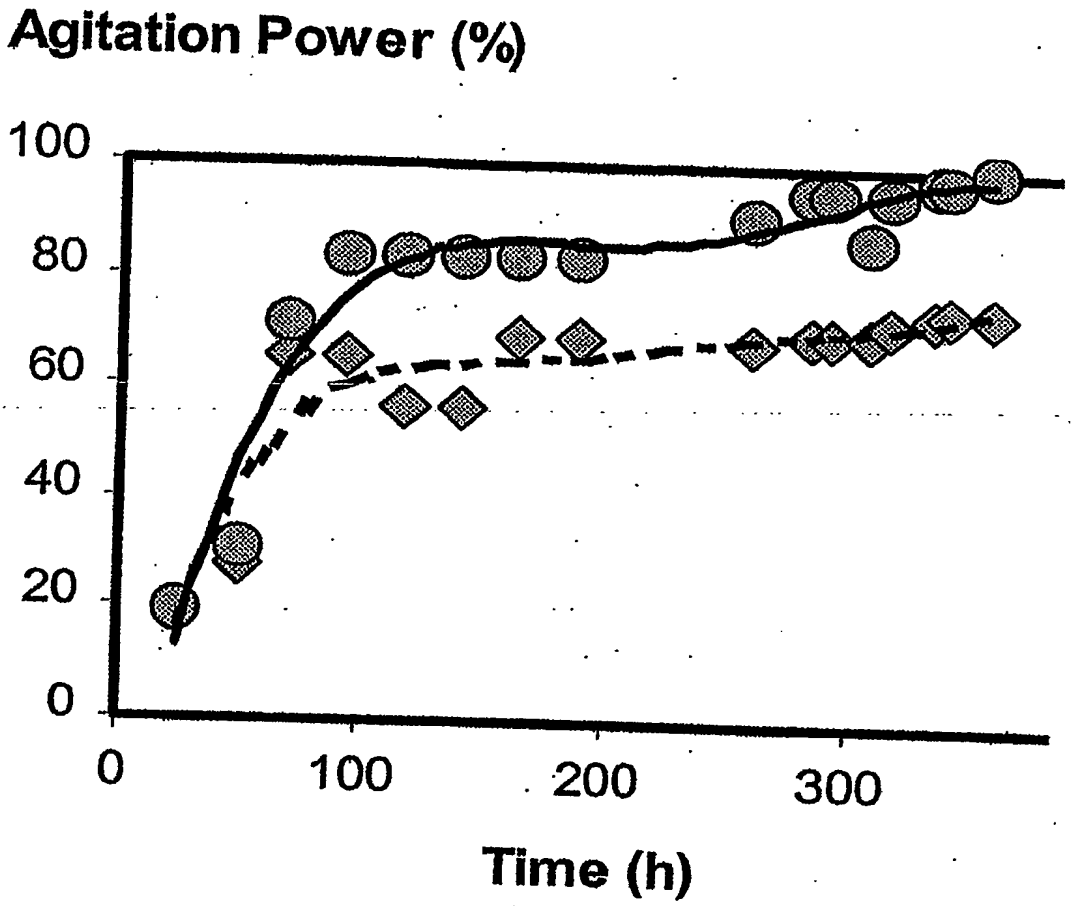

Fig. 2

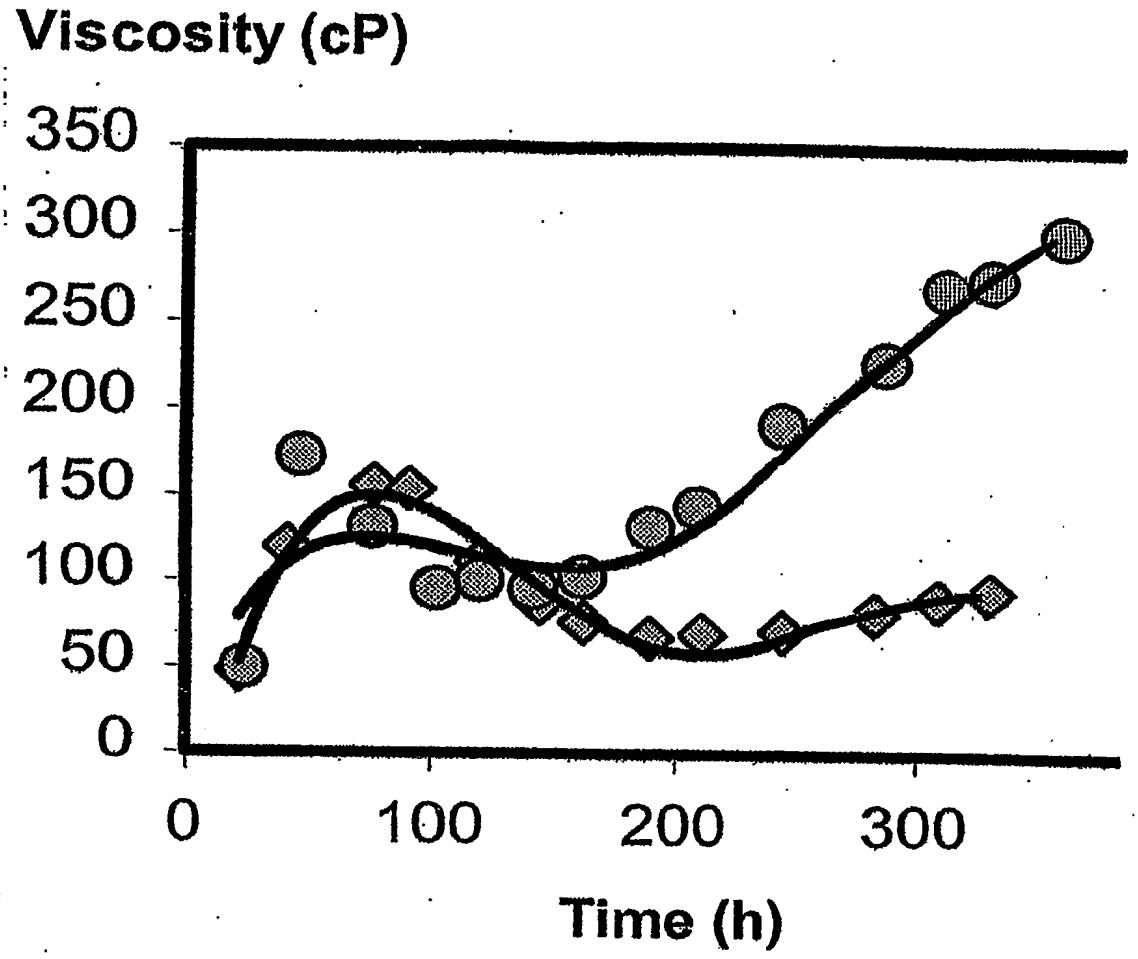

Fig. 3

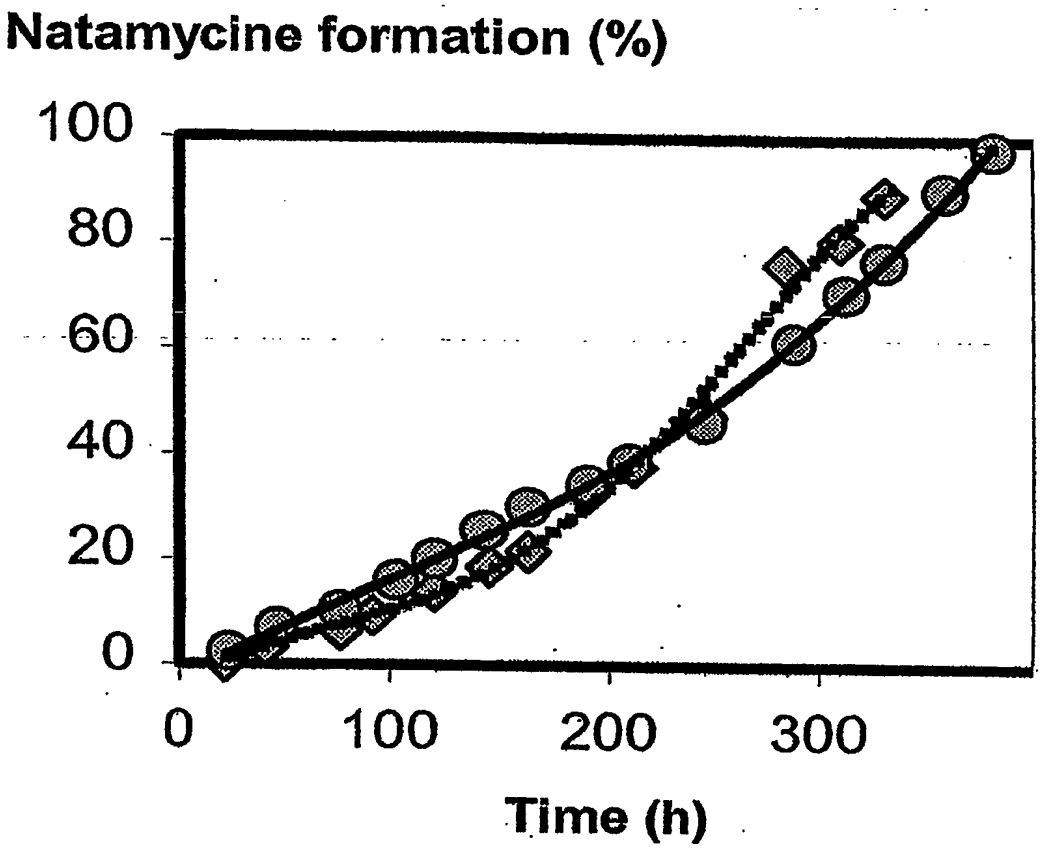

Fig. 4

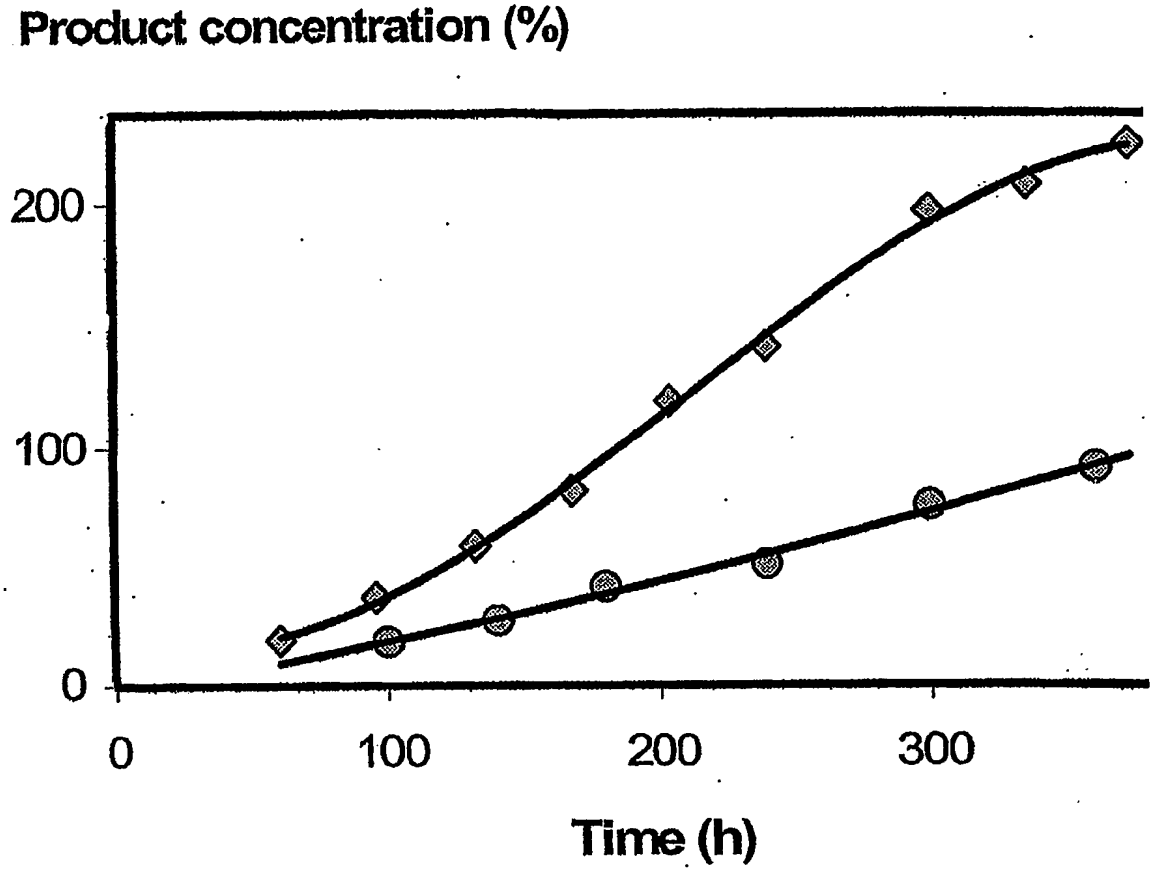

Fig.5